The Effects of De Novo Mutation on Gene Expression and the Consequences for Fitness in *Chlamydomonas reinhardtii*

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Abstract

Mutation is the ultimate source of genetic variation, the bedrock of evolution. Yet, predicting the consequences of new mutations remains a challenge in biology. Gene expression provides a potential link between a genotype and its phenotype. But the variation in gene expression created by de novo mutation and the fitness consequences of mutational changes to expression remain relatively unexplored. Here, we investigate the effects of >2,600 de novo mutations on gene expression across the transcriptome of 28 mutation accumulation lines derived from 2 independent wild-type genotypes of the green algae *Chlamydomonas reinhardtii*. We observed that the amount of genetic variance in gene expression created by mutation (V_m) was similar to the variance that mutation generates in typical polygenic phenotypic traits and approximately 15-fold the variance seen in the limited species where V_m in gene expression has been estimated. Despite the clear effect of mutation on expression, we did not observe a simple additive effect of mutation on expression change, with no linear correlation between the total expression change and mutation count of individual MA lines. We therefore inferred the distribution of expression effects of new mutations to connect the number of mutations to the number of differentially expressed genes (DEGs). Our inferred DEE is highly L-shaped with 95% of mutations causing 0-1 DEG while the remaining 5% are spread over a long tail of large effect mutations that cause multiple genes to change expression. The distribution is consistent with many *cis*-acting mutation targets that affect the expression of only 1 gene and a large target of *trans*-acting targets that have the potential to affect tens or hundreds of genes. Further evidence for *cis*-acting mutations can be seen in the overabundance of mutations in or near differentially expressed genes. Supporting evidence for *trans*-acting mutations comes from a 15:1 ratio of DEGs to mutations and the clusters of DEGs in the co-expression network, indicative of shared regulatory architecture. Lastly, we show that there is a negative correlation with the extent of expression divergence from the ancestor and fitness, providing direct evidence of the deleterious effects of perturbing gene expression.

Key words: **gene expression, mutation, evolution, *Chlamydomonas reinhardtii*.**

Introduction

Mutations provide both the raw variation for evolution through adaptation and contribute toward disease and aging. Unfortunately, a challenge in predicting the effects of mutations is linking the genotypic consequences of a mutation to phenotypic change. Gene expression provides a potentially useful link where we can observe changes in the regulation of genes due to mutation. Moreover, there is a longstanding hypothesis that evolution may proceed primarily through alteration in gene expression rather than changes in protein structure (King and Wilson 1975). This implies that the rate or distribution of fitness effects of regulatory mutations must be different from structural mutations to facilitate adaptive evolution of gene expression. Though there are now numerous examples of regulatory changes driving both adaptive changes (e.g. Abzhanov et al. 2006) and harmful diseases (e.g. Kleinjan and van Heyningen 2005), we still lack a general description of how variation in gene expression originates from de novo mutation and the distribution of effects of these mutations on fitness.

Comparative research has revealed numerous patterns in gene expression variation that provide insights into the impact of mutation on gene regulation (reviewed in Hill et al. 2021). In general, essential genes, genes typically with high expression, and those that are central in molecular interaction networks tend to display reduced expression variation within and between species (Jeong et al. 2001; Pál et al. 2001; Yu et al. 2004; Hahn and Kern 2005; He and Zhang 2006; Liao et al. 2006; Yu et al. 2007; Zotenko et al. 2008). An obvious explanation is that stabilizing selection on expression level removes deleterious variants and reduces the overall segregating expression variation. However, the distribution of mutational effects on expression (DEE) may also influence the extent of

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Discoveries

variation in different gene classes if mechanisms have evolved that buffer essential genes from perturbations by mutation. It has been observed that within species variation is driven by *trans*-regulatory variants, defined by regulatory mutations that affect distal genes. In contrast, *cis*-regulatory mutations, which affect proximal genes, underlie between-species variation (Wittkopp et al. 2004, 2008; Graze et al. 2009; Signor and Nuzhdin 2018). This trend might be explained if *trans*-regulatory mutations are more common, due to a larger mutation target. The more genes affected, the increasing likelihood of reducing fitness (Zande et al. 2022). With this in mind, we may also expect *trans*-regulatory variants to be kept at low frequencies and thus not contribute to between species divergence. Critically, the above explanations all depend on the rate, target size, expression effect, and fitness impact of regulatory mutations, highlighting the importance of studying the effect of mutation on expression.

Some of the most detailed information on how de novo mutation affects gene expression comes from experiments using single gene models where the effect of mutations on a reporter gene can be studied in detail. For example, in *Saccharomyces cerevisiae*, *cis*-acting mutations in the *TDH3* promoter were shown to generally reduce expression and have a greater effect on expression than *trans*-acting mutations, which did not show an asymmetry in the direction of effect (Metzger et al. 2016). However, although *trans*-acting mutations were relatively weak, they were 265 times more likely to occur than *cis*-acting mutations, implying a much larger mutational target (Gruber et al. 2012; Metzger et al. 2016). Despite the valuable insights gained from such precise experiments, these systems cannot capture the diverse mechanisms that may govern the effects of spontaneous mutations on expression throughout the transcriptome. For example, some genes have redundant promoters and regulatory proteins that maintain expression in the presence of mutational perturbations (reviewed by Payne and Wagner 2015; Signor and Nuzhdin 2018). Network feedback may also serve to mute the effects of mutation on the expression of genes, but such effects would likely be most evident when genes native to the genome in question are investigated. Additionally, there is evidence that *trans*-acting eQTLs may be clustered in hotspots, affecting thousands of genes (Albert et al. 2018), which are not observable in single gene model systems. There is therefore good reason to investigate the effect of de novo mutation on the transcriptome.

Investigating spontaneous mutations has been constrained by the rarity with which new mutations arise. Mutation accumulation (MA) experiments overcome this limitation by evolving lines under minimal selection, therefore facilitating the buildup of spontaneous changes irrespective of their fitness (Muller 1928). MA has been used extensively in the estimation of the rate, spectrum, and fitness effects of mutations. However, there are surprisingly few studies investigating the expression changes in MA lines in a small number of model organisms including *S. cerevisiae*, *Drosophila melanogaster*, and *Caenorhabditis elegans* (Denver et al. 2005; Rifkin et al. 2005; Landry et al. 2007; McGuigan et al. 2014; Huang et al. 2016; Zalts and Yanai 2017; Deiss et al. 2021). A key parameter in measuring how mutation affects gene expression is the contribution of mutation to expression variation per generation i.e. mutational variance (V_m) (Lynch and Walsh 1998). From the studies formerly mentioned, the mutational variance of *C. elegans* (Denver et al. 2005) was up to 9-fold higher than that of *D. melanogaster* (Rifkin et al. 2005) and yeast (Landry et al. 2007). MA studies have also shown that mutation introduced far more variation in expression than would be expected based on standing expression variation in natural populations, suggesting strong stabilizing selection in nature (Lemos et al. 2004; Denver et al. 2005; Rifkin et al. 2005; Huang et al. 2016). We have also learned that the expression of genes likely involved in fitness-related traits and pivotal developmental stages are highly constrained (Zhang and Li 2004; Rifkin et al. 2005; Zalts and Yanai 2017). Moreover, there is evidence of an inverse correlation between V_m and expression level (Rifkin et al. 2005). Despite these insights (Hine et al. 2018; Hodgins-Davis et al. 2019), our current knowledge of the global effects of mutations across the transcriptome is restricted to a small number of organisms with sometimes limited technologies and often in the absence of information about the underlying genomic mutations.

Here, we investigate the effect of de novo mutation on gene expression and fitness in *Chlamydomonas reinhardtii*. This haploid green alga is a longstanding model for numerous aspects of cell, molecular, and genome biology (Harris 2001; Grossman et al. 2003) and has emerged as a model for studying the evolution of mutation in eukaryotes. Over the past decade, numerous studies have explored mutation rate (Ness et al. 2012; Sung et al. 2012; Ness et al. 2015, 2016; Flynn et al. 2018), base spectrum, and the predictors of mutational variation across the genome of *C. reinhardtii* and related species (Ness et al. 2015; López-Cortegano et al. 2021). We also know that, on average, mutations in *C. reinhardtii* are deleterious, but evidence from the genetic mapping of mutational effects demonstrates a significant fraction of spontaneous mutations are beneficial (Morgan et al. 2014; Böndel et al. 2019). However, in this system, it remains unexplored how the accumulation of mutations has altered patterns of gene expression. Here, we sequenced the transcriptomes of 28 well-studied MA lines derived from 2 independent wild-type genotypes with fully characterized mutations and known fitness (Morgan et al. 2014; Ness et al. 2015; Böndel et al. 2019). Each MA line was grown for 800 to 1,100 generations and carries approximately 76 mutations. Integrating new transcriptome data with detailed characterization of these lines, we address the following questions: (i) How much does de novo mutation influence the direction and overall variation in gene expression? (ii) What is the distribution of mutational effects on expression? (iii) What are the properties of genes that are more or less robust to gene regulation perturbation by mutation? (iv) Do MA lines with more transcriptome divergence from mutation also suffer greater fitness declines?

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Methods

Sample Collection and Growth

We examined gene expression in 28 mutation accumulation lines derived from 2 wild-type *C. reinhardtii* strains: CC-2391 (n = 13) and CC-2344 (n = 15) (Morgan et al. 2014; Ness et al. 2015; Böndel et al. 2019). These MA lines have been intensively studied, including the characterization of the location of each mutation, the relative fitness of each MA line with respect to their unmutated ancestral line, the mutation rate of each line, and the distribution of fitness effects of all mutations. The MA lines were grown for 800 to 1,100 generations and carry a mean of 76.2 mutations in CC-2344 and 112.1 mutations in CC-2931. For the analyses presented here we focus primarily on single nucleotide mutations (SNMs) and small indels (<50 bp). Eight of the lines have also recently been characterized for larger structural mutations and we present an analysis of these mutations separately. To measure expression change from de novo mutation, we grew 3 replicates of each MA line and their unmutated ancestors synchronously on Bold's agar medium at 25 °C under standard, uniform laboratory conditions. Each replicate was grown until the density of colonies was sufficient for RNA isolation, then frozen at −80 °C until extraction. RNA extraction was conducted via the Maxwell RSC 48 Instrument and assessed for degradation, purity, and quantity using gel electrophoresis and a Qubit fluorometer. mRNA isolation and library preparation were conducted with the NEB mRNA stranded library preparation kit and Illumina NovaSeq preparation. All samples were sequenced using Illumina 100 bp paired-end sequencing with at least 20 M high-quality reads per sample. Sequencing and library preparation were performed by Genome Quebec.

Sequence Read Filtering and Quality Control

Sequence quality of raw reads was assessed with FastQC (v0.11.9) (Andrews 2010) and aligned using STAR aligner (v2.7.8a) (Dobin et al. 2013). We used the *C. reinhardtii* v6.0 reference genome with the mt+ mating type allele, and organelle genomes included (Craig et al. 2022). STAR alignment was conducted in a 2-pass mode, where the first pass identified possible splice junctions (SJ), and the second pass used the reference gene annotation in conjunction with the SJ output. Parameters of both alignments included "–alignIntronMax 5000" to exclude known false large introns identified in other STAR-mapped BAM files and "–outFilterMismatchNoverLmax 0.2", which allowed 0.2 × 100 bp mismatches for each single read to account for *C. reinhardtii*'s high genetic diversity (∼3%). The resulting BAM files were sorted and indexed with samtools (Li et al. 2009). Sequence coverage and mapping quality were measured with bamqc and rnaseq from the Qualimap quality control package (v2.2.2a) (Okonechnikov et al. 2016). Lastly, transcript quantification was done with featurecounts (v2.0.0) (Liao et al. 2013).

Differentially Expressed Gene (DEG) Calling

Read count normalization was conducted by DESeq2 (v1.30.1) (Love et al. 2014) using the median of ratios method to account for differing sequencing depth across samples. Low read counts were filtered by DESeq2 using the mean of normalized read counts across all samples within a strain (i.e. ancestors and replicates) as a filter statistic that optimizes statistically significant differentially expressed gene calling. We retained ∼95% of all genes across samples. We found a high expression correlation between the biological replicates of all samples using FPKM (fragments per kilobase of exon per million) normalized read counts of each gene (Pearson's $R > 0.96$, $P < 1.0 \times 10^{-4}$). Of the 90 replicates, only 2 had a lower correlation with their respective replicates (CC-2344 L6 replicate 2, Pearson's R ∼0.78, $P < 1.0 \times 10^{-4}$; CC-2931 L3 replicate 3, Pearson's R ∼0.80, $P < 1.0 \times 10^{-4}$). These replicates were removed from all analysis dependent on averaged normalized read counts, but all replicates were kept for calling differentially expressed genes (DEGs) given DESeq2 accounts for variance among replicates. DEGs in each MA line were identified with respect to its unmutated ancestral line with DESeq2. DESeq2 uses the Wald's test, $P_{adj} < 0.05$ for DEG calling, and the Benjamini–Hochberg method to account for multiple testing (FDR < 0.05). Across all MA lines, we identified 23,557 DEGs in CC-2344 (mean ∼1,571) and 15,829 DEGs in CCC-2391 (mean ∼1,218).

Quantifying Expression Variance Introduced by De Novo Mutation

To estimate the mutational variance, we used a generalized linear mixed model with a Poisson distribution to find the between line variation across MA lines.

$$\ln(Y_{ijk}) = \beta_1 \text{generation}_{ij} + u_{1i}\sqrt{\text{generation}_{ij}} + b_k + \ln\left(\frac{1}{\text{size factor } i}\right) + \varepsilon_{ijk}. \quad (1)$$

The model was fitted for each gene separately, where the between line variance per generation is given by the variance of u_{1i} ($2\text{Var}(u_{1i})$ ∼ mutational variance, V_m) (Lynch and Walsh 1998). The MA line was treated as a random effect $u_{1i} \sim N(0, \sigma^2_u)$ to account for per line variation given that the MA lines evolved independently of each other. Since we know the total number of mutations in each line, we were able to estimate the increase in expression variation per de novo mutation (V_{mm}) by substituting the number of generations with the mutation count in equation 1. In our model, Y_{ijk} is the raw read count of gene j, line i, and replicate k, which is normalized by a size factor $\ln(\frac{1}{\text{size factor}})$ to consider the varying sequencing depths across samples. Though we found a strong correlation between replicates, we also included an observation level random effect $b_k \sim N(0, \sigma^2_b)$ to account for overdispersion in our data. To estimate the mutational heritability $h^2_m = \frac{V_m}{V_e}$, the environmental variance (V_e) for each gene is given by the residual variance of the random slope $\sim\sigma^2_\epsilon$. For subsequent analysis, all variance estimates were $\log_2$ transformed to facilitate comparison with previous studies.

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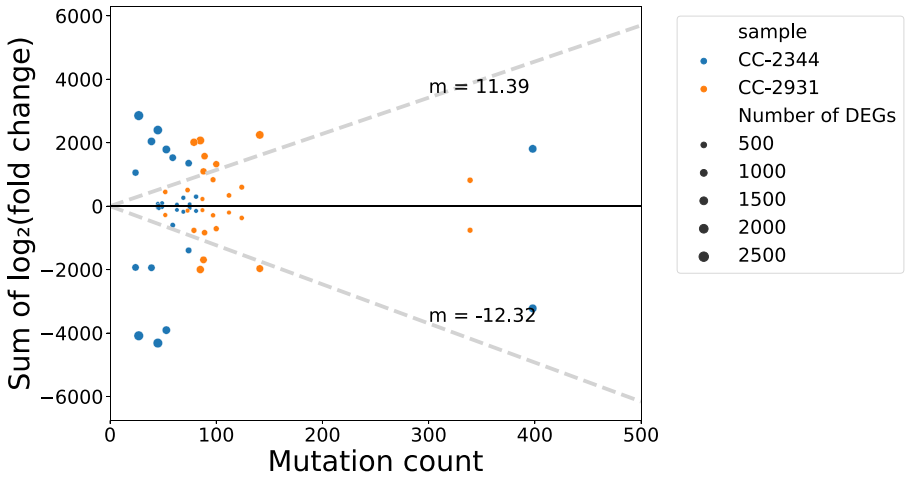

Fig. 1. No correlation between total expression change and mutation count. Plot of the total expression change of differentially expressed genes in each MA line against mutation count. Genes with positive and negative $\log_2$-fold changes were summed separately and their total expression change was represented as points above and below the black horizontal line. The dashed line represents the average directional rate of 2-fold expression change per mutation. There is no correlation between expression change in either direction and the number of mutations (negative: Pearson's $R = 0.1$, $P = 0.625$; positive: Pearson's $R = 0.08$, $P = 0.702$).

To test whether lines with more mutations demonstrated larger expression divergence from the unmutated ancestor, we quantified the $\log_2$-fold change of gene expression for each gene in each MA line relative to its respective unmutated ancestor. We then summed these expression changes for all significant DEGs ($P_{adj} < 0.05$) for each MA line and plotted it against the mutation count of the respective line to get an overall measure of divergence (Fig. 1). The DEGs in each MA line were divided into positive and negative expression change bins to consider any bias in the direction of change and avoid the canceling out of expression changes.

Inference of the Distribution of Expression Effects (DEE) of Novel Mutations

The number of DEGs generated by mutation is a function of the distribution of expression effects of new mutations. Given that many mutations likely have no effect on expression, while other mutations may affect many genes (reviewed in Emerson and Li 2010), we inferred the distribution of expression effects of mutations (DEE) by fitting a model to the number of DEGs caused by mutation in each of the 28 MA lines. We first fit a model where the number of DEGs caused by a mutation was drawn from a gamma distribution:

$$X \sim \text{Gam}(\theta, k),$$

where X is the number of DEGs and θ and k are the parameters for the shape and scale, respectively. We also reasoned that if many mutations caused 0 DEGs, a better fitting model could be used. This model could allow a fraction of mutations to cause 0 DEGs while other mutations would introduce 1 or more DEGs drawn from a gamma distribution shifted by 1. To find the parameters that best optimize the likelihood of seeing all 28 mutations-DEG counts in a given distribution, we use the SciPy global minima tool dual annealing, which was amended to find the global maximum instead of the minimum (Virtanen et al. 2020). The tool was run for 1 million iterations under varying temperatures to get a good estimate of the optimum. For the modified distribution, we split the bounds of no-effect mutations into 0.2 intervals and ran the model for each interval. The model of best fit was determined through Akaike and Bayesian Information criteria.

Correlation of Mutation Count and Total DEG Sum

To determine the expected correlation of mutation count and DEG count under our inferred DEE, we conducted 10,000 iterations of simulated data. We used the same number of mutations observed in each MA line ($n = 28$) to draw mutations from the inferred DEE to determine how many DEGs would have resulted. For each simulated trial, we found the correlation of the mutation and total DEG number.

Effect of Structural Variants on Expression

The structural variants (SVs) for 8 of our lines were previously identified by López-Cortegano et al. (2023). The location of the SV was based on the previous version of the reference genome (v5.0), which had some structural differences from the latest v6.0 reference of *C. reinhardtii*. These genomic changes led to the splitting of SV during the liftover of coordinates across reference genomes. The SVs were merged according to the proximity of their fragments and shared genomic features and the new coordinates of the majority of mutations were confirmed through IGV. To determine the frequency at which SV and DEGs colocalized and the likelihood of this occurrence, for each MA line, we found the proportion of genes overlapped by observed SV that were identified as one of n observed DEGs and compared it to those retrieved from 1,000 iterations of the same protocol performed with n randomized DEGs.

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Correlates of Expression Change

To test whether expression change depends on transcription level in the ancestral genotype, we categorized the genes into 10 percentile bins according to the FPKM-normalized read counts of the ancestral line. The lowest bin represents genes with the lowest read counts and those in the highest percentile bins consist of high expression genes. Each bin contains 1,745 and 1,736 genes for CC-2344 and CC-2931, respectively. Next, we plotted the distribution of expression change within each percentile bin by taking the median $\log_2$-fold change of each gene across all MA lines within a strain.

Using genes in the metabolic network (iCre1355), we tested whether the position of genes in the network, specifically their connectedness to other genes predicted their robustness to mutational perturbations. The degree centrality (i.e. indegree, outdegree, and betweenness centrality) of each gene was estimated (Imam et al. 2015) from the iCre1355 network after conversion to a gene centric network (Chaiboonchoe et al. 2016). iCre1355 was constructed using the *C. reinhardtii* v5 gene annotation with 1,355 metabolic genes, and after liftover to the v6 annotation, 1,304 metabolic genes remained.

We also tested whether genes central in the co-expression network were buffered against expression change due to mutation. We identified hub genes in the Strenkert et al. (2019) co-expression network that measured the expression of the transcriptome at 2 h intervals over a 24 h period. Using the $\log_2$(FPKM + 1) normalized expression data retrieved from the Strenkert et al. (2019) supplementary, we generated an adjacency matrix with a soft threshold of 2 using WGCNA (Langfelder and Horvath 2008; Langfelder and Horvath 2012). The adjacency matrix was subsequently transformed into a topological overlap matrix which was used to cluster coexpressed genes using hierarchical clustering analysis. Of the 33 resultant clusters, those that were correlated by 0.75 were merged to provide a total of 13 distinct clusters. A total of 99.9% of genes were placed within these 13 co-expression modules. Hub genes were identified as genes with the top 5% intramodular connectivity per module. There were 890 hub genes based on the v5 gene annotation, which translated to 774 hub genes in v6. We measured the relationship between expression change and intramodular/total connectivity using multiple regression and compared the median expression change in hub genes against non-hub genes.

Evidence of Cis- and Trans-acting Mutations

To identify putative *cis* mutations, we measured the number of mutations located within 100 bp of the nearest DEG. Given the possibility of random chance neighboring mutation-DEG pairs, we use a permutation test to identify the presence of significant mutation-DEG clustering. We compared the number of nearby mutations to *n* observed DEGs per MA line to a null distribution generated by 10,000 iterations of nearby mutations to *n* randomly selected DEGs. The *P* value, the fraction of simulated DEGs with a higher number of mutation-DEG clustering than our real dataset, was subsequently compared to $\alpha = 0.05$ to test for the presence of potential *cis* mutations.

To group DEGs that might arise from mutations in common regulatory elements, we considered whether DEGs were clustered in the *Chlamydomonas* co-expression network (Strenkert et al. 2019). To test for excess clustering, we calculated the shortest path in a network between DEGs in the same MA line using the multi_source_dijkstra function in the NetworkX python package (Hagberg et al. 2008). Paths between genes (i.e. edges) in the co-expression network represent correlations in gene expression. Since genes can be connected through hubs, we measured total connectedness by converting each correlation coefficient (weight of the path) to path length using the equation: distance = correlation coefficient^{-1}. We then took the sum of all paths between 2 genes to get the total distance between 2 DEGs in the co-expression network. The shortest total paths represent the strongest correlations. We generated a null distribution that accounts for the fact that higher expression genes are more likely to be significantly differentially expressed. We took *n* random genes with similar expression levels as the observed DEGs, where *n* is the number of DEGs in a given MA line, and found the shortest path lengths between those *n* genes. Genes of similar expression were defined by partitioning the ancestral read counts into 5 percentile bins. Bins containing high expression genes were broken into smaller intervals given the heavily right skewed distribution of the read counts. We then conducted a permutation test to compare the distribution of shortest path lengths between observed DEGs for each MA line against the null distribution. Lines with signs of significant clustering had shorter median distances than their simulated counterparts. A *P* value was assigned as the fraction of trials where simulated DEGs had equal or shorter path lengths than the observed data. The function multi_source_dijkstra from NetworkX was amended to exclude unconnected DEGs from the analysis.

Fitness of Mutation Accumulation Lines

The fitness of all 28 MA lines was previously characterized in Morgan et al. (2014). Fitness was measured as the mean of maximum growth rate from 2 independent assays in Bold's medium. To assess fitness change, all fitness measures were scaled relative to the growth rate of the unmutated ancestor. To test for an association of fitness change with expression change, we performed a linear regression of relative fitness against the number of DEGs per line. We performed a similar regression using absolute-fold expression change which resulted in qualitatively similar results (supplementary fig. S6, Supplementary Material online).

Results and Discussion

Mutational Variance

In this study, we examined how >2,600 mutations across 28 MA lines have altered gene expression in the transcriptome

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of *C. reinhardtii*. On average, each MA line had 1,407 differentially expressed genes (DEGs), with more DEGs in MA lines of strain CC-2344 (mean 1,571 DEGs/MA line) than CC-2931 (mean 1,218 DEGs/MA line). Across all MA lines 23,557 DEGs were called with DESeq2 which uses the Wald's test, $P_{adj} < 0.05$ and the Benjamini–Hochberg method to account for multiple testing (FDR < 0.05). We first estimated mutational variance (V_m) (Lynch and Walsh 1998), which describes the change in expression variation introduced into each gene in each generation due to mutation. Measured as a $\log_2$-fold change in RNASeq read count, we found that the median V_m across genes was 8.2×10^{-4} and 6.1×10^{-4} for CC-2344 and CC-2931, respectively. Similar to the mean number of DEGs, the V_m per gene was higher in MA lines derived from CC-2344. The 1.3-fold elevation in CC-2344 was unexpected given that it has a lower mutation rate than CC-2931 (Ness et al. 2015). Our estimates of V_m for gene expression were on average 1.6 to 6× that of *C. elegans* (Denver et al. 2005; Hodgins-Davis et al. 2019; Deiss et al. 2021) and 6 to 15× that of *S. cerevisiae* (Landry et al. 2007; Hodgins-Davis et al. 2019). However, it is difficult to directly compare V_m between different organisms due to numerous factors including varying experimental designs, inclusion of all genes versus only DEGs, and technological differences between microarray and RNASeq. Even including these estimates, it is very clear from the within and between species variation in V_m that there is a compelling reason for similar studies in a much broader array of organisms.

Our estimates of V_m also permit calculation of mutational heritability ($h^2_m \sim \frac{V_m}{V_e}$, where V_e is the environmental variance) which is useful in comparing how much variation mutation can generate across different traits. We observed a median mutational heritability of per gene expression as 1.4×10^{-3} across the 28 MA lines from 2 strains. A recent review by Conradsen et al. (2022) showed that h^2_m ranged widely from 2.5×10^{-5} to 1.0×10^{-2} across numerous traits and taxa. It is challenging to compare across studies as both the organisms and the environmental variance will strongly influence heritability estimates. Regardless, our estimate of h^2_m for gene expression fell in the higher end of this range, and is broadly comparable to the amount of variation generated by mutation in other largely polygenic traits. Notably, microbes and transcriptomic traits were excluded from the review, but the fact that the h^2_m of gene expression in *C. reinhardtii* was within the range of other traits implies that mutation can create a substantial amount of variation for natural selection to act upon. As *C. reinhardtii* is haploid, the effects of mutations cannot be masked by heterozygosity, which may contribute to higher estimates of h^2_m. One might assume that the mutational target size of a single gene's expression is relatively low compared to complex life history traits or fitness, but if there are numerous *trans*-acting effects caused by regulatory network feedback this may not be the case. The P_{TDH3}-YFP reporter gene in yeast was estimated to have a *trans* mutational target size of 118 kbp (Metzger et al. 2016). Assuming the *C. reinhardtii* single base mutation rate of 1.2×10^{-9} and 900 generations (Ness et al. 2015), this target size implies that 1 in 7.7 genes should experience a regulatory mutation during the MA experiment, and some of these mutations may affect multiple genes.

Mutational variance is often scaled per generation, partly to facilitate estimates of trait evolution over time, but also because the number of mutations causing the change in each line is often unknown. As mutations are the cause of expression change and the expected number of mutations per generation varies between individuals and across species, we may gain insight from per-mutation estimates of mutational variance. Here, we were able to use mutation counts for each line to estimate expression change per mutation (V_{mm}) using generalized mixed-effect linear modeling. Across genes, we found a 4.9× difference in median V_{mm} of the strains. These values are both substantially higher than the V_m per generation, which is predicted given that we only expect ∼1 mutation per 10 generations in *C. reinhardtii*. However, while the direction of change between mutation variance scaled per generation and per mutation is expected, the magnitude is much larger than expected based on the number of mutations per generation (V_{mm} is 243× V_m). This discrepancy may in part be due to the evolution of mutator strains among the MA lines, with 3.5× to 8× higher mutation rates than other MA lines from the same strain. When we excluded these 2 mutators from our model, V_{mm} estimates dropped by more than half (CC-2344 $V_{mm} = 0.15$; CC-2931 $V_{mm} = 0.028$), but not enough to explain the discrepancy between V_m and V_{mm}. Taken together, the lack of congruence between the mutation rate of the 2 strains and the amount of mutation variance for expression, as well as the relatively larger V_{mm} to V_m estimates, suggests the relationship between mutation count and the extent of expression divergence is complex, which we explore more below.

Distribution of Expression Effects of Mutation

Contrary to the simple prediction that more mutations would introduce greater changes in expression, we found no linear correlation between total absolute $\log_2$-fold expression change and the number of mutations in a MA line (supplementary fig. S1, Supplementary Material online, $R^2 = 0.058$, $P = 0.421$). In fact, some MA lines with very few mutations (CC-2344) had the most DEGs and total change in expression (Fig. 1). We found a strong positive relationship between the number of DEGs and absolute transcriptomic change ($R^2 = 0.989$, $P < 1 \times 10^{-4}$) and a range of effect sizes among the DEGs. To investigate whether there was a bias in the direction of expression change, we separated genes into up- and downregulated categories. Lines that experienced large expression changes tended to accumulate a similar amount of expression change in both directions (Pearson's $R = 0.826$, $P = 6.03 \times 10^{-8}$). It is likely that there are more ways for mutation to damage *trans*- or *cis*-acting regulators

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reducing their ability to drive expression, but compromised repressors could increase expression. It is also possible that many of the observed changes result from feedbacks in the regulatory network such that some pathways or genes increase in expression in response to changes decrease in others. To ensure that the pattern between mutation count and expression change was not driven by large fold changes in genes with ancestrally low expression, we also considered the relationship between mutation and read count differences (MA—ancestral read count), but similarly found no correlation (supplementary fig. S2, Supplementary Material online).

One explanation for the weak linear correlation that we see between expression change and mutation count is that the underlying distribution of expression effects of mutation (DEE) includes a large number of mutations with little or no effect and a long tail of rare mutations that drive large changes in expression. We therefore attempted to infer the DEE as a gamma distribution that would describe the number of DEGs seen in each of the 28 MA lines as a function of the number of mutations they each harbor. We fit 3 models, 1 gamma distribution, and 2 where the gamma was shifted with a preset fraction of mutations that introduced 0 DEGs or 0 and 1 DEG. Of the 3 models, the best fitting distribution was strongly right skewed with 95% of mutations causing 0-1 DEGs. According to this model with a discrete bin of zero-effect mutations plus a gamma, 42% of mutations caused no change in expression, 53% caused 1 DEG, and 5% caused 2 or more genes to alter in expression (Fig. 2). The long tail of high effect mutations meant that the overall mean number of DEGs per mutation is 14.3. Although we likely do not have enough data to precisely infer the shape of the DEE for mutations of large effect (those causing 2 or more DEGs), the 3 models reach a consensus. Many mutations have no effect on expression, a fraction of mutations affect a single gene, while the long tail of large effect mutations has the potential to create many DEGs. However, when we simulated the number of DEGs that would be expected for 28 MA lines with the observed number of mutations, the correlation between mutation number and DEG count was stronger in the simulated than in the observed data. The fact that the observed data has a weaker than expected correlation between mutation and DEG count implies that there could be nonadditive effects where mutations are interacting or compensating the effects of other mutations. This is best exemplified by a mutator line with 3.6 fold higher mutation count than the mean but only 0.7× the mean number of DEGs. A more comprehensive model could incorporate the strength and direction of expression and potentially higher order interactions, but we were limited in power by the number of MA lines. Nevertheless, our model fits with what we know about the effects of *cis*- and *trans*-regulatory mutations with *cis* mutations affecting only local genes, while *trans*-acting mutations have the potential to alter the expression of many genes through pleiotropy (Wittkopp 2023). The existence of a small fraction of large effect mutation also potentially explains why some MA lines with very few mutations have very divergent transcriptomes while others with more mutations have relatively few DEGs.

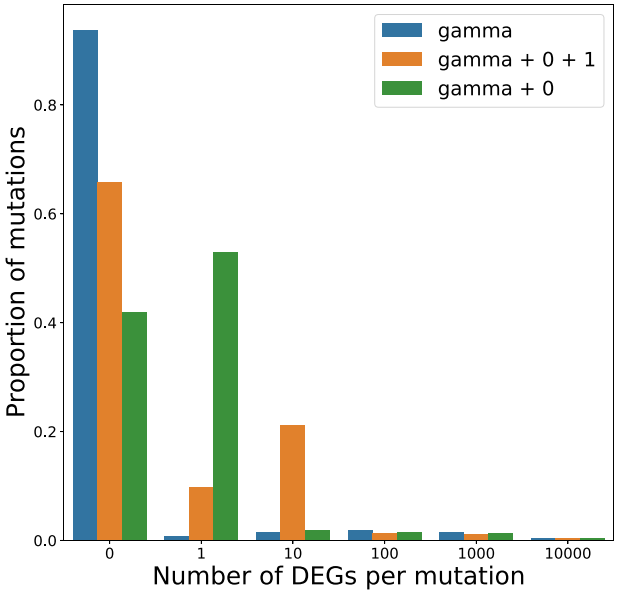

Fig. 2. Distribution of expression effects of de novo mutations. The inferred distributions describe the number of differentially expressed genes caused by a randomly selected de novo mutation. Three distributions were used to fit the mutation and DEG counts of all 28 MA lines. Of the 3 distributions, the best fitting model included a category of mutations that cause no differential expression (0 DEG) and a gamma distribution shifted by 1. The mean number of DEGs per mutation was 14.3 but 95% of mutations were estimated to change the expression of 0 or 1 gene, while the remaining 5% caused 2 or more (0-DEG category = 0.42, gamma shape = 0.0118, gamma scale = 2,005).

Colocalization of Structural Mutations and DEGs

We took advantage of the recent characterization of spontaneous structural variation that was conducted in 8 of our CC-2931 MA lines (López-Cortegano et al. 2023). These 238 SVs consisted of 125 insertions (263 bp ± 1,523 bp), 14 deletions (12,405 bp ± 21,986 bp), 41 duplications (16,227 bp ± 21,052 bp), 18 excisions (9 bp ± 7 bp), 11 inversions (1,504 bp ± 3,104 bp), and 29 translocations (10 bp ± 2 bp). None of these structural mutation types showed a strong bias in the direction of expression change outside of expectation. Unexpectedly, 1 of 9 deleted genes was upregulated, which we interpreted as either a misidentified translocated gene or perhaps an upregulated gene duplicate in CC-2931 that is not present in the ancestor. Ninety-six of the SVs overlapped a total of 161 genes, of which 39% were differentially expressed. The proportion of DEGs that colocalized with SVs ranged from 0.27 to 0.62 per MA line, which was a significant enrichment in every line relative to the null expectation of the random overlap of DEGs and SVs (permutation test, $P > 0.001$). These results clearly support that SVs played a significant role in driving expression changes of overlapping genes. This is not surprising given the potential for SVs to separate

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genes from their local context or to delete regions of the gene or its regulatory apparatus. Perhaps more unexpected was that many of the genes were upregulated. While this may be expected for duplications (82% upregulated), it is not expected for most of the other SV classes. For example, half of the 10 translocated genes were upregulated while the rest were downregulated. However, while SVs were clearly important for expression regulation, the number of SVs in a line did not correlate with the amount of expression divergence (Pearson's $R = -0.46$, $P = 0.25$).

Correlates of Expression Change

It is often hypothesized that genes with high expression are subject to stronger selective constraints either because of their adaptive significance or because of the harmful consequences of misfolding or mistranslation (Drummond et al. 2005; Zhang and Yang 2015). Indeed, in numerous species, there is a negative correlation between expression level and the rate of protein sequence evolution (Pál et al. 2001; Pál et al. 2003; Bloom and Adami 2004; Marais et al. 2004; Rocha and Danchin 2004; Zhang and Li 2004). We find a similar pattern in *C. reinhardtii* with high expression genes displaying reduced sequence divergence relative to *Caenorhabditis incerta* (0.12 for $K_a/K_{\text{shigh expression genes}} <$ 0.33 for $K_a/K_{\text{slow expression genes}}$, Brunner Munzel test, $P = 5.41 \times 10^{-104}$). It is also reasonable to posit that expression changes in high expression genes may be critical to the transcriptome and may have evolved robust expression regulation, making them less susceptible to mutational or environmental perturbation. Contrary to our predictions, we see relatively little evidence that ancestral expression level predicts the extent of expression change due to mutation (supplementary fig. S3, Supplementary Material online). Genes with higher ancestral expression in fact show a slight elevation in fold expression change from the ancestor. On the other hand, we find a clear correlation that higher expression genes have higher V_m and h_m^2; but this reflects the expected mean-variance correlation when V_m is estimated from read counts, such that genes with more reads have more scope to vary in absolute terms. Relatively few other studies have examined the relationship of expression level and susceptibility to mutation. Similar to our findings, there was an overall weak positive relationship of h_m^2 and expression observed in *D. melanogaster* (supplementary fig. S4, Supplementary Material online), but that was slightly negative when developmental stages were separated (Rifkin et al. 2005). So while our results support the finding that protein evolution is slower in high expression genes, we find no evidence that the mutational effects are buffered in these high expression genes.

In addition to expression level, the interactions among genes and their relative regulation can be summarized in interaction networks. The centrality–lethality rule describes the concept that highly connected genes in an interaction network are more likely to be critical to fitness (Jeong et al. 2001; Yu et al. 2004; Hahn and Kern 2005; He and Zhang 2006; Yu et al. 2007; Zotenko et al. 2008). There is clear support for this idea from experimental and comparative genomics (Pál et al. 2001; Rocha and Danchin 2004; Subramanian and Kumar 2004; Zhang and He 2005; Liao et al. 2006; Zhang and Yang 2015). Extending this idea, it is reasonable that the expression level of central genes is also subject to stronger stabilizing selection, and that mechanisms may have evolved to protect central genes from mutations that alter their expression. Using a reconstruction of *C. reinhardtii*'s metabolic network, iCre1355 (Imam et al. 2015), we tested whether highly connected metabolic genes (i.e. degree and betweenness centrality) underwent less expression change due to mutation. Contrary to our prediction, we found that the genes with the highest degree (those with the most connections to other genes) had a 25% higher median absolute-fold expression change than the lowest degree genes (low degree genes = 0.35, high degree genes = 0.425, Brunner Munzel test, $P < 0.03$). We also noted that the subset of genes included in the metabolic network had higher expression than the genome average and therefore tended to have higher V_m because of the same mean-variance correlation of V_m and expression noted above. When we look for altered patterns of expression change in the regulatory network, we find a similarly significant positive correlation of connectivity (i.e. intramodular connectivity) in a regulatory module with the median expression change of a gene although the amount of variation explained is relatively low ($R^2 = 0.04$, $P = 0.001$). The positive relationship between connectivity and expression divergence does not support the idea that mechanisms have evolved to buffer these genes from mutational effects, but nor does it rule it out. Given the interconnectedness of hub genes, it may be expected that they would be more prone to perturbation and the fact that their mutation vulnerability is quite similar to other genes could be a consequence of these buffering mechanisms. No previous mutation accumulation studies have examined the relationship between network topology and mutational effects on expression. However, in *D. melanogaster* (Lemos et al. 2004, 2005), reduced divergence and polymorphism of expression level were seen in highly connected proteins, and if this trend holds in *Chlamydomonas*, it would imply that there is stronger stabilizing selection removing the incoming variation from mutation.

Evidence of Cis- and Trans-acting Mutations

The inferred DEE suggested a large fraction of mutations that cause only 1 or a small number of DEGs, which could represent mutations in *cis*-acting regulatory regions that affect local genes. We therefore sought to test for an overrepresentation of mutations near DEGs, under the simplifying assumption that *cis* mutations are near the genes they affect. To generate a null distribution of the expected distance of a DEG to its nearest mutation, we conducted 10,000 iterations where the relative positions of mutations and DEGs were randomized. To reflect the nonuniform distribution of mutations, we retained their position in

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the genome and randomly assigned genes as DEGs. The null was constructed in this way to ensure that we could discern an enrichment of DEGs near mutation from more mutations near genes in general. When we compared the distribution created with observed DEGs to the null, we found an enrichment of DEGs with mutations within 100 bp of the gene flanks in 12 of 28 MA lines (permutation test, $P < 0.05$; supplementary fig. S5, Supplementary Material online) and an additional 6 lines with marginally significant P values (permutation test, $0.05 < P < 0.1$). In total, we found 243 potential *cis* mutations (~9%). In Fig. 3, we display a representative sample (CC-2344 L14) showing evidence of an overabundance of mutations near DEGs (red) relative to the expectation if there was no relationship (blue). We also found several mutations within DEGs, which were twice as likely to be found in UTRs as would be expected based on the proportion of UTR sequences in the genome. This may be expected given that UTRs are enriched with promoters/enhancers and are known to regulate RNA transcription, stability, and translation (Steri et al. 2018). We also noticed that MA lines with many DEGs were less likely to show the enrichment of nearby mutations, hence the lack of putative *cis* mutations in some MA lines. This may be due to the presence of 1 or more *trans*-acting mutations with multiple regulatory targets that masked the signals of any *cis*-acting mutations that only affect 1 gene. Though previous experimental results suggest that *cis*-acting mutations tend to downregulate genes (Metzger et al. 2016), we did not see a bias in the direction of expression change in DEGs that were <100 bp from a mutation. From single gene reporter systems (Metzger et al. 2016), we have estimates that the *trans*-acting mutation target is 385-fold larger than the *cis*-target for that single reporter gene. However, if *cis*-acting mutations are gene-specific, and *trans*-acting mutations are pleiotropic, then the full DEE of all genes might have a larger overall proportion of target sites for *cis*-acting mutations and could reconcile why we see a relatively clear signal of mutations near genes.

Although the majority of the inferred DEE is small effect mutations, the long tail of mutations causing multiple DEGs may indicate the presence of *trans*-acting sites with pleiotropic effects. It is clear from the ratio of DEGs to mutations (mean 15.7 DEGs/mutation) that there are many mutations that affect multiple genes, especially given that many mutations will have little or no effect on expression. We also found that DEGs tended to be clustered near one another in the *Chlamydomonas* co-expression network (Strenkert et al. 2019). The median shortest path between pairs of DEGs from the same MA line was less than the median shortest path length between random pairs of genes in 15 out of 28 MA lines (permutation test, $P < 0.05$). Of the 15 lines, we could only detect putative *cis* mutations in 8 given the obscured signal in MA lines with many DEGs. Figure 4 is a representative plot of 1 MA line (CC-2344 L1), where the signal of co-expression network clustering can be observed as an excess of short paths between DEGs. A similar pattern was seen in *C. elegans* (Denver et al. 2005) where gene expression changes from mutation accumulation clustered in the network, and were interpreted as evidence of *trans*-acting mutations. It has been shown in *S. cerevisiae* that the

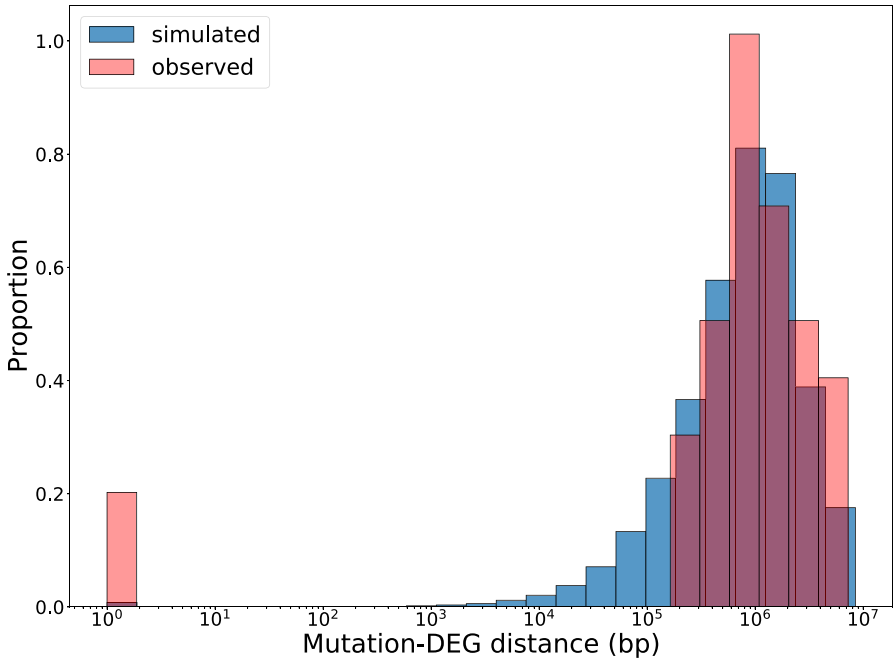

Fig. 3. Evidence of *cis*-regulatory mutations. Distribution of distance between a DEG and its nearest mutation for observed and simulated data. The observed distribution is one representative MA line and the expected distribution is based on 10,000 trials where the positions of DEGs in the genome are randomized. The excess of DEGs co-localizing with mutations is apparent in the excess of observed data on the left of the distribution. Mutations found within the coding or noncoding regions (UTRs and introns) were assigned a distance of 1.

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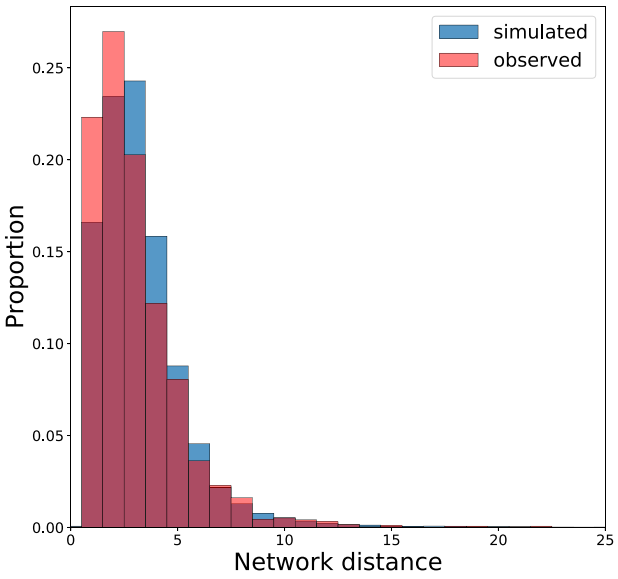

Fig. 4. Clustering of DEGs in the co-expression network. Overlaid distributions of the shortest path lengths between observed DEGs and the null expectation. The null distribution was created by calculating the shortest paths among a random sample of genes from the co-expression network. The figure above is a representative plot of a single MA line demonstrating an excess of observed short paths among DEGs at the left of the plot indicative of clustering (permutation test, $P < 0.05$). Fifteen of 28 MA lines had a significant excess of clustered DEGs.

topology of the co-expression network can explain the pleiotropic effects of *trans*-acting mutations, in line with what we observe. Significant clustering of DEGs could be the consequence of mutation in a common *trans*-acting factor that controls expression in a given co-expression module or regulatory feedbacks that result from alterations to the expression or structure of related genes.

Fitness Consequences of Expression Change

It has been shown that expression is often under stabilizing selection (Denver et al. 2005; Rifkin et al. 2005; Huang et al. 2016), thus we would expect MA lines with more total expression change to equally experience more fitness decline. Consistent with this prediction, we found a negative correlation between the number of DEGs per MA line and fitness (Fig. 5, $R^2 = 0.233$, $P = 0.032$), and a similar relationship between the sum of absolute $\log_2$-fold change and fitness (supplementary fig. S6, Supplementary Material online; $R^2 = 0.2$, $P = 0.034$). It is worth noting that the correlation was dependent on a single influential data point with high expression divergence and low fitness at the bottom right of Fig. 5 (excluding this data point, $R^2 = 0.183$, $P = 0.201$). However, there is nothing otherwise unusual about this particular data that would justify its exclusion, so while the relationship is not strong, more replication would be required to determine if the relationship between fitness and expression is real. The association between declining fitness and increasing number of DEGs could imply the deleterious nature of large changes in the transcriptome due to mutation. However, the

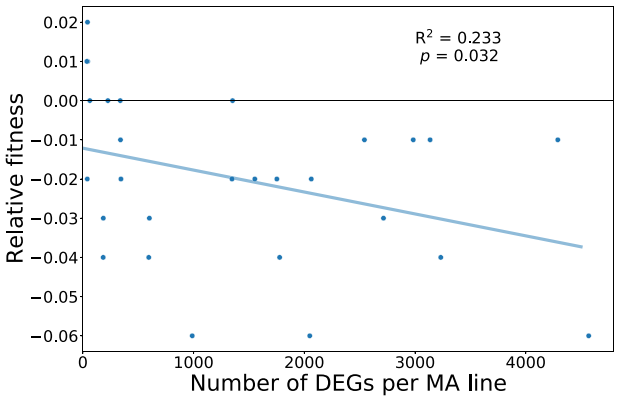

Fig. 5. Evidence of fitness decline in lines with more differentially expressed genes. Linear regression modeling the negative relationship between the number of significant differentially expressed genes in each MA line and its fitness relative to the unmutated ancestor ($R^2 = 0.233$, $P = 0.032$).

direction of causality is difficult to assign, as it is also possible that deleterious mutations alter the biology of the cell so strongly that other genes change expression as part of a stress response or some other regulatory cascade. Zande et al. (2022) had similar findings in yeast, where *trans* mutations had negative fitness effects due to pleiotropy. It is also worth noting that these same lines have previously been analyzed, where it was seen that there was a negative relationship between the number of mutations and fitness (Kraemer et al. 2017; Böndel et al. 2019, 2022). But, given that we did not see a relationship between the amount of expression divergence and mutation count, the contribution of some deleterious mutations may have been to non-expression related traits while others influenced expression to cause an overall decline in fitness. In combination with our results which describe the effects of a random set of de novo mutations, evidence suggests that *C. reinhardtii* is vulnerable to harmful mutations that disrupt the regulation of gene expression.

Taken together, we see clear evidence that mutations can generate a substantial amount of genetic variation in gene expression and that this new variation is important for organismal performance. For example, it has been a long-held belief that the contribution of mutation to genetic variance is less than 1% of the environmental variance. However, we found that mutation may contribute significantly more to variation per generation than formerly considered, almost 24%. After accounting for the larger contribution of environmental variance under natural settings, this remains a considerable amount of mutational variation that is heritable. It may change the perception of the raw materials available for evolution via selection to act upon. Using mutation accumulation lines allows us to observe the cumulative effect of mutations from the spectrum of naturally occurring mutations. From this, we estimated a distribution of expression effects that suggested a substantial mutational target for large effect mutations that pleiotropically generate expression changes across the transcriptome. There are some lines harboring tens of mutations showing

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thousands of differentially expressed genes. It seems likely that these pleiotropic mutations are driven not only by mutations in *trans*-acting regulators like transcription factors, but also by alterations in the balance of the metabolism of the cell and feedbacks in the regulatory network. Despite this, we see no evidence for mutational robustness in genes central to the metabolic and co-expression network. A limitation of our data is that we cannot link the expression changes to individual mutations and therefore cannot assess the nature of these large effect mutations. Therefore, the possibility of compensatory mutations means that some expression change may be masked by subsequent mutations. With current technology, it is not cost-effective to map *trans*-acting mutations to their expression effects but advances in single cell technologies may allow for such resolution in the near future. Though we failed to identify the expression consequence of each mutation, we did find that in 8 of our lines, 39% of genes affected by structural mutation were differentially expressed. Surprisingly, excluding those mutations that removed or added copies of genes, these structural variants were equally likely to up- or down-regulate affected genes.

Supplementary Material

Supplementary material is available at *Molecular Biology and Evolution* online.

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Data Availability

The code used for the analysis can be found at https://github.com/enibalogun/The-effect-of-mutation-on-geneexpression.git and the raw data at the European Genome-Phenome Archive (PRJEB51708/ERP136361).

References

Abzhanov A, Kuo WP, Hartmann C, Grant BR, Grant PR, Tabin CJ. The calmodulin pathway and evolution of elongated beak morphology in Darwin's finches. *Nature*. 2006:**442**(7102):563–567. https://doi.org/10.1038/nature04843.

Albert FW, Bloom JS, Siegel J, Day L, Kruglyak L. Genetics of *trans*-regulatory variation in gene expression. *Elife*. 2018:**7**:e35471. https://doi.org/10.7554/eLife.35471.

Andrews Simon. FastQC: a quality control tool for high throughput sequence data [Internet]. 2010. Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/.

Bloom JD, Adami C. Evolutionary rate depends on number of protein–protein interactions independently of gene expression level: response. *BMC Evol Biol*. 2004:**4**(1):14. https://doi.org/10.1186/1471-2148-4-14.

Böndel KB, Kraemer SA, Samuels T, McClean D, Lachapelle J, Ness RW, Colegrave N, Keightley PD. Inferring the distribution of fitness effects of spontaneous mutations in *Chlamydomonas reinhardtii*. *PLoS Biol*. 2019:**17**(6):e3000192. https://doi.org/10.1371/journal.pbio.3000192.

Böndel KB, Samuels T, Craig RJ, Ness RW, Colegrave N, Keightley PD. The distribution of fitness effects of spontaneous mutations in *Chlamydomonas reinhardtii* inferred using frequency changes under experimental evolution. *PLoS Genet*. 2022:**18**(6):e1009840. https://doi.org/10.1371/journal.pgen.1009840.

Chaiboonchoe A, Ghamsari L, Dohai B, Ng P, Khraiwesh B, Jaiswal A, Jijakli K, Koussa J, Nelson DR, Cai H, *et al*. Systems level analysis of the *Chlamydomonas reinhardtii* metabolic network reveals variability in evolutionary co-conservation. *Mol Biosyst*. 2016:**12**(8):2394–2407. https://doi.org/10.1039/C6MB00237D.

Conradsen C, Blows MW, McGuigan K. Causes of variability in estimates of mutational variance from mutation accumulation experiments. *Genetics*. 2022:**221**(2):iyac060. https://doi.org/10.1093/genetics/iyac060.

Craig RJ, Gallaher SD, Shu S, Salomé P, Jenkins JW, Blaby-Haas CE, Purvine SO, O'Donnell S, Barry K, Grimwood J, *et al*. The *Chlamydomonas* Genome Project, version 6: reference assemblies for mating type plus and minus strains reveal extensive structural mutation in the laboratory. *Plant Cell*. 2022:**35**(2):644–672. https://doi.org/10.1093/plcell/koac347.

Deiss TC, Konrad A, Bergthorsson U, Katju V. 2021. Global gene expression divergence in spontaneous mutation accumulation lines of *Caenorhabditis elegans* under varying efficiency of selection. bioRxiv 442498. https://www.biorxiv.org/content/10.1101/2021.05.03.442498, 04 May 2021, preprint: not peer reviewed.

Denver DR, Morris K, Streelman JT, Kim SK, Lynch M, Thomas WK. The transcriptional consequences of mutation and natural selection in *Caenorhabditis elegans*. *Nat Genet*. 2005:**37**(5):544–548. https://doi.org/10.1038/ng1554.

Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*. 2013:**29**(1):15–21. https://doi.org/10.1093/bioinformatics/bts635.

Drummond DA, Bloom JD, Adami C, Wilke CO, Arnold FH. Why highly expressed proteins evolve slowly. *Proc Natl Acad Sci U S A*. 2005:**102**(40):14338–14343. https://doi.org/10.1073/pnas.0504070102.

Emerson JJ, Li W-H. The genetic basis of evolutionary change in gene expression levels. *Philos Trans R Soc Lond B Biol Sci*. 2010:**365**(1552):2581–2590. https://doi.org/10.1098/rstb.2010.0005.

Flynn JM, Lower SE, Barbash DA, Clark AG. Rates and patterns of mutation in tandem repetitive DNA in six independent lineages of *Chlamydomonas reinhardtii*. *Genome Biol Evol*. 2018:**10**(7):1673–1686. https://doi.org/10.1093/gbe/evy123.

Graze RM, McIntyre LM, Main BJ, Wayne ML, Nuzhdin SV. Regulatory divergence in *Drosophila melanogaster* and *D. simulans*, a genomewide analysis of allele-specific expression. *Genetics*. 2009:**183**(2):547–561; 1SI – 21SI. https://doi.org/10.1534/genetics.109.105957.

Grossman AR, Harris EE, Hauser C, Lefebvre PA, Martinez D, Rokhsar D, Shrager J, Silflow CD, Stern D, Vallon O, *et al*. *Chlamydomonas reinhardtii* at the crossroads of genomics. *Eukaryot Cell*. 2003:**2**(6):1137–1150. https://doi.org/10.1128/EC.2.6.1137-1150.2003.

Gruber JD, Vogel K, Kalay G, Wittkopp PJ. Contrasting properties of gene-specific regulatory, coding, and copy number mutations in *Saccharomyces cerevisiae*: frequency, effects, and dominance.

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PLoS Genet. 2012:**8**(2):e1002497. https://doi.org/10.1371/journal.pgen.1002497.
Hagberg A, Swart P, Chult D. Exploring network structure, dynamics, and function using networkx. Los Alamos National Lab. (LANL), Los Alamos, NM (United States) 2008. Available from: https://www.osti.gov/biblio/960616
Hahn MW, Kern AD. Comparative genomics of centrality and essentiality in three eukaryotic protein-interaction networks. *Mol Biol Evol*. 2005:**22**(4):803–806. https://doi.org/10.1093/molbev/msi072.
Harris EH. *Chlamydomonas* as a model organism. *Annu Rev Plant Physiol Plant Mol Biol*. 2001:**52**(1):363–406. https://doi.org/10.1146/annurev.arplant.52.1.363.
He X, Zhang J. Why do hubs tend to be essential in protein networks? *PLoS Genet*. 2006:**2**(6):e88. https://doi.org/10.1371/journal.pgen.0020088.
Hill MS, Vande Zande P, Wittkopp PJ. Molecular and evolutionary processes generating variation in gene expression. *Nat Rev Genet*. 2021:**22**(4):203–215. https://doi.org/10.1038/s41576-020-00304-w.
Hine E, Runcie DE, McGuigan K, Blows MW. Uneven distribution of mutational variance across the transcriptome of *Drosophila serrata* revealed by high-dimensional analysis of gene expression. *Genetics*. 2018:**209**(4):1319–1328. https://doi.org/10.1534/genetics.118.300757.
Hodgins-Davis A, Duveau F, Walker EA, Wittkopp PJ. Empirical measures of mutational effects define neutral models of regulatory evolution in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci U S A*. 2019:**116**(42):21085–21093. https://doi.org/10.1073/pnas.1902823116.
Huang W, Lyman RF, Lyman RA, Carbone MA, Harbison ST, Magwire MM, Mackay TF. Correction: spontaneous mutations and the origin and maintenance of quantitative genetic variation. *Elife*. 2016:**5**:e22300. https://doi.org/10.7554/eLife.22300.
Imam S, Schäuble S, Valenzuela J, López García de Lomana A, Carter W, Price ND, Baliga NS. A refined genome-scale reconstruction of *Chlamydomonas* metabolism provides a platform for systems-level analyses. *Plant J*. 2015:**84**(6):1239–1256. https://doi.org/10.1111/tpj.13059.
Jeong H, Mason SP, Barabási AL, Oltvai ZN. Lethality and centrality in protein networks. *Nature*. 2001:**411**(6833):41–42. https://doi.org/10.1038/35075138.
King MC, Wilson AC. Evolution at two levels in humans and chimpanzees. *Science*. 1975:**188**(4184):107–116. https://doi.org/10.1126/science.1090005.
Kleinjan DA, van Heyningen V. Long-range control of gene expression: emerging mechanisms and disruption in disease. *Am J Hum Genet*. 2005:**76**(1):8–32. https://doi.org/10.1086/426833.
Kraemer SA, Böndel KB, Ness RW, Keightley PD, Colegrave N. Fitness change in relation to mutation number in spontaneous mutation accumulation lines of *Chlamydomonas reinhardtii*. *Evolution*. 2017:**71**(12):2918–2929. https://doi.org/10.1111/evo.13360.
Landry CR, Lemos B, Rifkin SA, Dickinson WJ, Hartl DL. Genetic properties influencing the evolvability of gene expression. *Science*. 2007:**317**(5834):118–121. https://doi.org/10.1126/science.1140247.
Langfelder P, Horvath S. WGCNA: an R package for weighted correlation network analysis. *BMC Bioinformatics*. 2008:**9**(1):559. https://doi.org/10.1186/1471-2105-9-559.
Langfelder P, Horvath S. Fast R functions for robust correlations and hierarchical clustering. *J Stat Softw*. 2012:**46**(11):i11. https://doi.org/10.18637/jss.v046.i11.
Lemos B, Bettencourt BR, Meiklejohn CD, Hartl DL. Evolution of proteins and gene expression levels are coupled in *Drosophila* and are independently associated with mRNA abundance, protein length, and number of protein–protein interactions. *Mol Biol Evol*. 2005:**22**(5):1345–1354. https://doi.org/10.1093/molbev/msi122.
Lemos B, Meiklejohn CD, Hartl DL. Regulatory evolution across the protein interaction network. *Nat Genet*. 2004:**36**(10):1059–1060. https://doi.org/10.1038/ng1427.
Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R; 1000 Genome Project Data Processing Subgroup. The Sequence Alignment/Map format and SAMtools. *Bioinformatics*. 2009:**25**(16):2078–2079. https://doi.org/10.1093/bioinformatics/btp352.
Liao B-Y, Scott NM, Zhang J. Impacts of gene essentiality, expression pattern, and gene compactness on the evolutionary rate of mammalian proteins. *Mol Biol Evol*. 2006:**23**(11):2072–2080. https://doi.org/10.1093/molbev/msl076.
Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics*. 2013:**30**(7):923–930. https://doi.org/10.1093/bioinformatics/btt656.
López-Cortegano E, Craig RJ, Chebib J, Balogun EJ, Keightley PD. Rates and spectra of de novo structural mutations in *Chlamydomonas reinhardtii*. *Genome Res*. 2023:**33**(1):45–60. https://doi.org/10.1101/gr.276957.122.
López-Cortegano E, Craig RJ, Chebib J, Samuels T, Morgan AD, Kraemer SA, Böndel KB, Ness RW, Colegrave N, Keightley PD. *De novo* mutation rate variation and its determinants in *Chlamydomonas*. *Mol Biol Evol*. 2021:**38**(9):3709–3723. https://doi.org/10.1093/molbev/msab140.
Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol*. 2014:**15**(12):550. https://doi.org/10.1186/s13059-014-0550-8.
Lynch M, Walsh B. Genetics and analysis of quantitative traits 1998. Available from: http://www.invemar.org.co/redcostera1/invemar/docs/RinconLiterario/2011/febrero/AG_8.pdf
Marais G, Domazet-Lošo T, Tautz D, Charlesworth B. Correlated evolution of synonymous and nonsynonymous sites in Drosophila. *J Mol Evol*. 2004:**59**(6):771–779. https://doi.org/10.1007/s00239-004-2671-2.
McGuigan K, Collet JM, McGraw EA, Ye YH, Allen SL, Chenoweth SF, Blows MW. The nature and extent of mutational pleiotropy in gene expression of male *Drosophila serrata*. *Genetics*. 2014:**196**(3):911–921. https://doi.org/10.1534/genetics.114.161232.
Metzger BPH, Duveau F, Yuan DC, Tryban S, Yang B, Wittkopp PJ. Contrasting frequencies and effects of *cis*- and *trans*-regulatory mutations affecting gene expression. *Mol Biol Evol*. 2016:**33**(5):1131–1146. https://doi.org/10.1093/molbev/msw011.
Morgan AD, Ness RW, Keightley PD, Colegrave N. Spontaneous mutation accumulation in multiple strains of the green alga, *Chlamydomonas reinhardtii*. *Evolution*. 2014:**68**(9):2589–2602. https://doi.org/10.1111/evo.12448.
Muller HJ. The Measurement Of Gene Mutation Rate In *Drosophila*, Its High Variability, And Its Dependence upon temperature. *Genetics*. 1928:**13**(4):279–357. https://doi.org/10.1093/genetics/13.4.279.
Ness RW, Kraemer SA, Colegrave N, Keightley PD. Direct estimate of the spontaneous mutation rate uncovers the effects of drift and recombination in the *Chlamydomonas reinhardtii* plastid genome. *Mol Biol Evol*. 2016:**33**(3):800–808. https://doi.org/10.1093/molbev/msv272.
Ness RW, Morgan AD, Colegrave N, Keightley PD. Estimate of the spontaneous mutation rate in *Chlamydomonas reinhardtii*. *Genetics*. 2012:**192**(4):1447–1454. https://doi.org/10.1534/genetics.112.145078.
Ness RW, Morgan AD, Vasanthakrishnan RB, Colegrave N, Keightley PD. Extensive *de novo* mutation rate variation between individuals and across the genome of *Chlamydomonas reinhardtii*. *Genome Res*. 2015:**25**(11):1739–1749. https://doi.org/10.1101/gr.191494.115.
Okonechnikov K, Conesa A, García-Alcalde F. Qualimap 2: advanced multi-sample quality control for high-throughput sequencing data. *Bioinformatics*. 2016:**32**(2):292–294. https://doi.org/10.1093/bioinformatics/btv566.
Pál C, Papp B, Hurst LD. Highly expressed genes in yeast evolve slowly. *Genetics*. 2001:**158**(2):927–931. https://doi.org/10.1093/genetics/158.2.927.
Pál C, Papp B, Hurst LD. Genomic function: rate of evolution and gene dispensability. *Nature*. 2003:**421**(6922):496–497; discussion 497–498. https://doi.org/10.1038/421496b.

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Payne JL, Wagner A. Mechanisms of mutational robustness in transcriptional regulation. *Front Genet*. 2015:**6**:322. https://doi.org/10.3389/fgene.2015.00322.

Rifkin SA, Houle D, Kim J, White KP. A mutation accumulation assay reveals a broad capacity for rapid evolution of gene expression. *Nature*. 2005:**438**(7065):220–223. https://doi.org/10.1038/nature04114.

Rocha EPC, Danchin A. An analysis of determinants of amino acids substitution rates in bacterial proteins. *Mol Biol Evol*. 2004:**21**(1):108–116. https://doi.org/10.1093/molbev/msh004.

Signor SA, Nuzhdin SV. The evolution of gene expression in *cis* and *trans*. *Trends Genet*. 2018:**34**(7):532–544. https://doi.org/10.1016/j.tig.2018.03.007.

Steri M, Idda ML, Whalen MB, Orrù V. Genetic variants in mRNA untranslated regions. *Wiley Interdiscip Rev RNA*. 2018:**9**(4):e1474. https://doi.org/10.1002/wrna.1474.

Strenkert D, Schmollinger S, Gallaher SD, Salomé PA, Purvine SO, Nicora CD, Mettler-Altmann T, Soubeyrand E, Weber APM, Lipton MS, *et al.* Multiomics resolution of molecular events during a day in the life of *Chlamydomonas*. *Proc Natl Acad Sci U S A*. 2019:**116**(6):2374–2383. https://doi.org/10.1073/pnas.1815238116.

Subramanian S, Kumar S. Gene expression intensity shapes evolutionary rates of the proteins encoded by the vertebrate genome. *Genetics*. 2004:**168**(1):373–381. https://doi.org/10.1534/genetics.104.028944.

Sung W, Ackerman MS, Miller SF, Doak TG, Lynch M. Drift-barrier hypothesis and mutation-rate evolution. *Proc Natl Acad Sci U S A*. 2012:**109**(45):18488–18492. https://doi.org/10.1073/pnas.1216223109.

Virtanen P, Gommers R, Oliphant TE, Haberland M, Reddy T, Cournapeau D, Burovski E, Peterson P, Weckesser W, Bright J, *et al.* SciPy 1.0: fundamental algorithms for scientific computing in Python. *Nat Methods*. 2020:**17**(3):261–272. https://doi.org/10.1038/s41592-019-0686-2.

Wittkopp PJ. Contributions of mutation and selection to regulatory variation: lessons from the *Saccharomyces cerevisiae TDH3* gene. *Philos Trans R Soc Lond B Biol Sci*. 2023:**378**(1877):20220057. https://doi.org/10.1098/rstb.2022.0057.

Wittkopp PJ, Haerum BK, Clark AG. Evolutionary changes in *cis* and *trans* gene regulation. *Nature*. 2004:**430**(6995):85–88. https://doi.org/10.1038/nature02698.

Wittkopp PJ, Haerum BK, Clark AG. Regulatory changes underlying expression differences within and between *Drosophila* species. *Nat Genet*. 2008:**40**(3):346–350. https://doi.org/10.1038/ng.77.

Yu H, Greenbaum D, Xin Lu H, Zhu X, Gerstein M. Genomic analysis of essentiality within protein networks. *Trends Genet*. 2004:**20**(6):227–231. https://doi.org/10.1016/j.tig.2004.04.008.

Yu H, Kim PM, Sprecher E, Trifonov V, Gerstein M. The importance of bottlenecks in protein networks: correlation with gene essentiality and expression dynamics. *PLoS Comput Biol*. 2007:**3**(4):e59. https://doi.org/10.1371/journal.pcbi.0030059.

Zalts H, Yanai I. Developmental constraints shape the evolution of the nematode mid-developmental transition. *Nat Ecol Evol*. 2017:**1**(5):113. https://doi.org/10.1038/s41559-017-0113.

Zande PV, Hill MS, Wittkopp PJ. Pleiotropic effects of *trans*-regulatory mutations on fitness and gene expression. *Science*. 2022:**377**(6601):105–109. https://doi.org/10.1126/science.abj7185.

Zhang J, He X. Significant impact of protein dispensability on the instantaneous rate of protein evolution. *Mol Biol Evol*. 2005:**22**(4):1147–1155. https://doi.org/10.1093/molbev/msi101.

Zhang L, Li W-H. Mammalian housekeeping genes evolve more slowly than tissue-specific genes. *Mol Biol Evol*. 2004:**21**(2):236–239. https://doi.org/10.1093/molbev/msh010.

Zhang J, Yang J-R. Determinants of the rate of protein sequence evolution. *Nat Rev Genet*. 2015:**16**(7):409–420. https://doi.org/10.1038/nrg3950.

Zotenko E, Mestre J, O'Leary DP, Przytycka TM. Why do hubs in the yeast protein interaction network tend to be essential: reexamining the connection between the network topology and essentiality. *PLoS Comput Biol*. 2008:**4**(8):e1000140. https://doi.org/10.1371/journal.pcbi.1000140.

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